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## REMARKS

Claims 98-102 stand rejected under 35 U.S.C. §101 as allegedly not supported by either a specific and/or substantial asserted utility or a well established utility. Claims 98-102 also stand rejected under 35 U.S.C. §112, first paragraph, as allegedly not supported by either a specific and/or substantial asserted utility or a well established utility so that one of ordinary skill in the art would allegedly not know how to use the claimed invention. Applicants respectfully traverse these rejections as discussed below.

Applicants have argued in previous communications that the teaching provided in the present application as filed, irrespective of any subsequent confirmatory data, and also in view of subsequent confirmatory data, meets the requirements of 35 U.S.C. §101 and the requirements of 35 U.S.C. §112, first paragraph, as discussed below. Applicants explicitly incorporate by reference and reiterate all previously made arguments with respect to the rejections of Claims 98-102 under 35 U.S.C. §101 and under 35 U.S.C. §112, first paragraph. Additional arguments are presented below.

### The Rejections of Claims 98-102 Under 35 U.S.C. §101

Claims 98-102 stand rejected under 35 U.S.C. §101 as allegedly not supported by either a specific and/or substantial asserted utility or a well established utility. Applicants respectfully traverse this rejection.

The Examiner states: "Therefore, because no known specific biological activity is described within the instant specification nor specifically associated with any nucleic acid that encodes the polypeptides of SEQ ID NO:17, because the specification merely discloses on page 55 that the human 'GFR$\alpha$3 does not bind any of these [GDNF family member] molecules (Figure 9C)', and that 'GFR$\alpha$3 is thus an orphan receptor', the claimed polynucleotides have no specific nor substantial utility because further experimentation is also necessary at the time of filing the instant invention to attribute a function and 'real world" utility to the claimed nucleic acid molecules" (this Office Action, page 6, first full paragraph).

Applicants respectfully disagree with the Examiner's characterization of the present invention, and submit that the Examiner failed to apply the proper legal standard when making the rejection.

**Utility – Legal Standard**

According to the Utility Examination Guidelines ("Utility Guidelines"), 66 Fed. Reg. 1092 (2001) an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility."

Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that is to be diagnosed.

The requirement of "substantial utility" defines a "real world" use, and derives from the Supreme Court's holding in *Brenner v. Manson*, 383 U.S. 519, 534 (1966) stating that "The basic *quid pro quo* contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility." In explaining the "substantial utility" standard, M.P.E.P. 2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, **any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient**, at least with regard to defining a "substantial" utility." (M.P.E.P. 2107.01, emphasis added.) Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement, set forth in M.P.E.P, 2107 II (B) (1) gives the following instruction to patent examiners: "If the applicant has asserted that the claimed invention is useful for

any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

Finally, the Utility Guidelines restate the Patent Office's long established position that any asserted utility has to be "credible." "Credibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record . . . that is probative of the applicant's assertions." (M.P.E.P. 2107 II (B) (1) (ii)) Such standard is presumptively satisfied unless the logic underlying the assertion is seriously flawed, or if the facts upon which the assertion is based are inconsistent with the logic underlying the assertion (Revised Interim Utility Guidelines Training Materials, 1999).

**Proper Application of the Legal Standard**

As discussed in previous responses, specific and substantial uses for the claimed nucleic acids, vectors, cells and processes are asserted in the specification, which also provides multiple examples of such utility. As discussed previously, the specification as filed asserts and/or demonstrates utility including, for example, cell activation, ligand screening, treatment of disorders of the peripheral nervous system using ligands to GFR$\alpha$3, and other uses.

The Examiner states that "because no known specific biological activity is described within the instant specification nor specifically associated with any nucleic acid that encodes polypeptides of SEQ ID NO: 17 ... the claimed polynucleotides have no specific nor substantial utility" (Office action, page 4, lines 17-21). However, as discussed more fully below, Applicants respectfully note that the specification does teach specific biological activity. For example, at pages 28, lines 34-38 and 29, lines 1-14, ligands to GFR$\alpha$3 are explicitly said to be useful for treatment of diseases or conditions of the peripheral nervous system. The specification teaches GFR$\alpha$3 ligands (see, *e.g.,* page 56, lines 23-38 to page 57, lines 1-13). Ligand-mediated activation of (chimeric) GFR$\alpha$3 receptors is disclosed (see, *e.g.,* page 55, lines 31-37 to page 56, lines 1-22; page 57, lines 15-38 to page 58, lines 1-32). The only conceivable reason

for the for the fact that the Examiner overlooks these utilities is that he is looking for a "currently available" use, instead of accepting Applicants' assertion of a series of credible utilities, as dictated by applicable case law and reflected in the Guidelines cited above.

***Specific, Credible and Substantial GFRα3 Utility is Disclosed in the Application***

The present application, and both provisional applications the priority of which is claimed, disclose specific, credible and substantial utility for the novel GFRα3 receptor disclosed in the application. Applicants submit that this utility was demonstrated by the disclosure and examples of the application as filed, and by its parent provisional applications. For example, U.S. Provisional Patent Application Serial Nos. 60/079,124 and 60/081569 discuss treatment of diseases or conditions of the peripheral nervous system on page 30, lines 1-24; the use of GFRα3 antibodies on pages 37, lines 2 to 29; chimeric receptors including GFRα3 at page 51, line 8 to page 52, line 26. In addition, Applicants note that support for the asserted utility of the claimed invention is also found in subsequent publications (some of which have been cited in the specification itself) and that such corroborative support further shows that the invention has real world utility.

The specification states, and it was recognized in the art at the time, that a receptor may be bound by a ligand; such binding is useful to activate the receptor, or to inhibit its action, to label it, and in other ways. The law does not require the identification of a natural ligand to establish utility for a receptor; for example, exogenous ligands (*e.g.,* opiates) or artificial ligands may usefully interact with a receptor and may serve to support a claim of utility. Indeed, Applicants use the term "ligand" in a broad sense, not limiting its meaning to a native ligand of GFRα3.

Thus, Applicants note that antibodies may be ligands: *i.e.,* may act as agonists or antagonists to receptors to activate, or to inhibit, the action of their target receptors. Antibody agonists were known at the time of filing the application (see, *e.g.,* Eur. J. Immunol. 27(5):1108-1114 (1997) Thilenius et al., Agonist antibody and Fas ligand

mediate different sensitivity to death in the signaling pathways of Fas and cytoplasmic mutants) and such agonists were explicitly discussed in the specification as filed (see, *e.g.,* page 57, lines 16-19). Applicants in fact demonstrated binding of ligands to (chimeric) GFR$\alpha$3 receptors (page 56, lines 3-22). The specification further teaches antibodies to GFR$\alpha$3 that act as GFR$\alpha$3 ligands. Thus, specific biological activity for the GFR$\alpha$3 receptor is taught in the application as filed.

Furthermore, as indicated by Baloh et al. (cited in the specification at page 56, lines 17-19) the natural ligand for GFR$\alpha$3 has been subsequently identified and named "artemin." Artemin binds both GFR$\alpha$3 and the chimeric receptor fusion protein gD-GFR$\alpha$3-Rse-gD (page 56, lines 17-19). The inventors disclose that ligands (such as antibodies) may be screened for agonist activity against these receptors (page 56, lines 19-22).

Moreover, such utility has been confirmed by subsequent publications. For example, a subsequent publication by one of the inventors (Development 128(10):3685-3695 (2001)) confirms that the novel receptor GFR$\alpha$3 is important in the generation, survival and growth of peripheral (sympathetic) neurons in culture. Thus, Applicants submit that the specific utility disclosed in the application is substantial and credible, as shown by the specific statements in the specification (see, *e.g.,* page 28, lines 34-38 to page 29, lines 1-14; page 56, line 23 to page 58, line 32) and in subsequent publications by the inventors and by others as discussed above. This later-published work supports the specific, substantial and credible utility as disclosed and asserted in the parent provisional applications and in the present application, and inures to the benefit of the present application. "Inurement involves a claim by an inventor that, as a matter of law, the acts of another person should accrue to the benefit of the inventor." Cooper v. Goldfarb, 154 F.3d 1321, 1331, 47 USPQ2d 1896, 1904.

Accordingly, Applicants submit that the specification as filed disclosed specific, substantial and credible utility for the novel GFR$\alpha$3 receptor discovered by the inventors.

***The GFRα3 Receptor has Inherent Utility***

Applicants further submit that, even were the above arguments not to be accepted by the Examiner, the GFRα3 receptor has inherent utility.

Applicants submit that, in the present case, it is useful to consider the analogous situation of anticipation by inherency. Recent case law in the area of inherent anticipation has held that if a person of ordinary skill in the art, presented with all facts, would understand that the missing structure, composition or function is always necessarily present in the cited prior art, a holding of anticipation by inherency is proper. It is not required that prior to the invention one skilled in the art recognized the presence of the inherent structure, composition or function. The objective understanding of the presence of the inherent structure, composition or function can occur later. Atlas Powder Co. v. Ireco Inc. 190 F.3d 1342; 51 USPQ2d 1943 (Fed. Cir. 1999); Schering Corporation v. Geneva Pharmaceuticals, Inc. 67USPQ2d 1664 (Fed. Cir 2003).

The GFRα3 receptor discovered and disclosed by the present inventors was described in the application as being useful for antibody formation, treatment of disorders of the peripheral nervous system (using ligands to GFRα3, such as antibody ligands), cell activation, production of chimeric receptors, ligand screening, and other uses. The GFRα3 receptor, by its nature, is effective to bind ligands (whether natural or otherwise) and to affect cells in which it is expressed. The asserted uses of the GFRα3 receptor, in large part, utilize such actions of the novel receptor. Since such actions are inherent in the receptor itself, then such uses are inherent in the receptor itself. Receptor activation or inhibition upon ligand binding was well known in the art at the time of filing the application. These actions being inherent in the receptor, disclosure of the GFRα3 receptor was effective to provide these asserted utilities to the public. These actions being inherent in the GFRα3 receptor, and being specific to that receptor, of the receptor, the specification discloses specific utility for the receptor. Since the effects of ligand binding on receptors was well known, such asserted utility would be credible. Such uses are substantial, providing such "real world" uses as

treatments of diseases or conditions of the peripheral nervous system. Accordingly, taken from the perspective of one of ordinary skill in the art, Applicants' disclosure provides a reasonable use that is specific, substantial and credible.

Thus, the ability of the novel GFR$\alpha$3 receptor discovered by the Applicants to bind and to be affected by ligands is inherent in the receptor itself, and was recognized as such by the Applicants (*e.g.*, page 56, lines 5-6 "an assay to identify agonist antibodies and a natural ligand (or other agonists) for mammalian GFR$\alpha$3 follows the method described above for GFR$\alpha$2-Rse"). The natural ligand was subsequently discovered, as noted in the specification (page 56, lines 17-18). The binding activity of the novel GFR$\alpha$3 receptor was disclosed to be useful for specific purposes (*e.g.*, page 30, lines 11-14, treatment of disorders of the peripheral nervous system).

Thus, where, as here, the Applicants have disclosed a novel receptor; have demonstrated activation of chimeric receptor constructs by non-natural ligands of the novel receptor; have stated that it has a natural ligand, and have disclosed the identity of the later-discovered natural ligand in the filed application itself; then, the expected identification of the natural ligand inures to the Applicants and serves to support the specific and/or substantial asserted utility or a well established utility as required by 35 U.S.C. § 101. Such support is in concert with, and in addition to, the other uses discussed previously and discussed above.

Applicants submit that the utility disclosed in the application, alone and when taken in view of subsequent publications, demonstrates a specific and/or substantial asserted utility or a well established utility as required by 35 U.S.C. §101. Accordingly, Applicants respectfully submit that the rejections of Claims 98-102 under 35 U.S.C. §101 are overcome.

**The Rejections of Claims 98-102 Under 35 U.S.C. §112, First Paragraph**

Claims 98-102 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly not being supported by either a specific and/or substantial asserted utility or a well

established utility so that one skilled in the art would not know how to use the claimed invention. Applicants respectfully traverse this rejection.

Applicants note that the specification includes multiple examples of the utility of the subject matter of Claims 98-102, as discussed above. Thus, the application provides several examples of specific and substantial utility for the present invention and of its use, and thereby teach one of ordinary skill in the art how to use the invention. Accordingly, Applicants respectfully submit that the rejections of Claims 98-102 under 35 U.S.C. §112, first paragraph, are overcome.

## CONCLUSION

Applicants respectfully submit that the rejections to Claims 98-102 are overcome by the above arguments. Accordingly, Applicants respectfully submit that Claims 98-102 stand in condition for allowance, and respectfully request the reconsideration and withdrawal of the rejections of Claims 98-102.

Please charge any fees, including the fees for extension of time, and any other fees due, or credit overpayment to Deposit Account No. **08-1641** referencing Attorney's Docket No. **39766-0065 A**.

Respectfully submitted,

Date: July 2, 2004

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# Multiple effects of artemin on sympathetic neurone generation, survival and growth

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## SUMMARY

**To define the role of artemin in sympathetic neurone development, we have studied the effect of artemin on the generation, survival and growth of sympathetic neurones in low-density dissociated cultures of mouse cervical and thoracic paravertebral sympathetic ganglia at stages throughout embryonic and postnatal development. Artemin promoted the proliferation of sympathetic neuroblasts and increased the generation of new neurones in cultures established from E12 to E14 ganglia. Artemin also exerted a transient survival-promoting action on newly generated neurones during these early stages of development. Between E16 and P8, artemin exerted no effect on survival, but by P12, as sympathetic neurones begin to acquire neurotrophic factor independent survival, artemin once again enhanced survival, and by P20 it promoted survival as effectively as nerve growth factor (NGF). During this late period of development, artemin also enhanced the growth of neurites from cultured neurones more effectively than NGF. Confirming the physiological relevance of the mitogenic action of artemin on cultured neuroblasts, there was a marked reduction in the rate of neuroblast proliferation in the sympathetic ganglia of mice lacking the GFRα3 subunit of the artemin receptor. These results indicate that artemin exerts several distinct effects on the generation, survival and growth of sympathetic neurones at different stages of development.**

Key words: Artemin, Sympathetic neurones, Mouse, Neurotrophins

## INTRODUCTION

Artemin (Baloh et al., 1998) is the most recently identified member of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors, which includes neurturin (Kotzbauer et al., 1996) and persephin (Milbrandt et al., 1998) in addition to the founding member GDNF (Lin et al., 1993). These secreted proteins promote the survival of various kinds of neurones of the peripheral nervous system (Suter-Crazzolara and Unsicker, 1994; Buj-Bello et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Wright and Snider, 1996; Molliver et al., 1997; Baloh et al., 1998; Heuckeroth et al., 1998; Forgie et al., 1999; Baudet et al., 2000) and central nervous system (Lin et al., 1993; Henderson et al., 1994; Arenas et al., 1995; Ha et al., 1996; Williams et al., 1996; Milbrandt et al., 1998) and play a role in the development of several other organs (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Moreover, some populations of neurones require different members of this family of proteins at different stages of their development; for example, parasympathetic neurones switch responsiveness from GDNF to neurturin during development (Forgie et al., 1999; Enomoto et al., 2000).

Members of the GDNF family use multicomponent receptors that consist of a common receptor tyrosine kinase signalling component Ret (Durbec et al., 1996; Trupp et al., 1996; Vega et al., 1996; Worby et al., 1996), plus one of a family of GPI-linked receptors (GFRα1 to 4) that confers ligand specificity. Studies of ligand binding, Ret phosphorylation and the responses of cells expressing these receptors have indicated that Ret/GFRα1 is the preferred receptor for GDNF (Klein et al., 1997; Jing et al., 1996; Treanor et al., 1996), Ret/GFRα2 is the preferred receptor for neurturin (Buj-Bello et al., 1997; Baloh et al., 1997; Creedon et al., 1997; Jing et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997; Widenfalk et al., 1997; Trupp et al., 1998), Ret/GFRα3 is the preferred receptor for artemin (Baloh et al., 1998) and Ret/GFRα4 is the receptor for persephin (Enokido et al., 1998).

The defective formation and maintenance of the sympathetic nervous system in mice with a disrupted *GFRα3* (*Gfra3* – Mouse Genome Informatics) gene (Nishino et al., 1999) prompted our present study to clarify the role of artemin in sympathetic neurone development. GFRα3-deficient mice survive to adulthood but exhibit the characteristic features of defective cranial sympathetic function. Although the number of cells in the superior cervical ganglion (SCG) of $GFR\alpha3^{-/-}$ embryos is apparently normal at E11.5 and continues to increase between E12.5 and birth, the number is only 60 to 80% of the number in wild-type SCG during this period of

embryonic development. The SCG is also displaced more caudally in *GFRα3*$^{-/-}$ embryos at E12.5 and later stages, possibly owing to failure of SCG precursors to complete the last step in their rostral migration from lower cervical levels (Nishino et al., 1999). It is unclear, however, whether this defect in precursor cell migration in GFRα3-deficient embryos is the cause of the reduced number of cells in the early SCG or whether artemin-GFRα3 signalling plays a role in sympathetic neuroblast proliferation or differentiation. By birth, the sympathetic innervation of several tissues is defective in *GFRα3*$^{-/-}$ mice, and during the postnatal period there is progressive death of neurones in the SCG so that by P60 fewer than 5% of the neurones remain (Nishino et al., 1999). It is unclear, however, whether this loss of SCG neurones is because sympathetic neurones depend on artemin for survival or whether the neurones fail to obtain other target-derived neurotrophic factors because artemin/GFRα3 signalling plays a key role in establishing sympathetic innervation. To shed light on these issues, we have examined the effects of artemin on sympathetic neurone generation, survival and growth in dissociated sympathetic ganglion cultures established over a broad range of ages from E12 to adulthood. We show that artemin promotes neuroblast proliferation and neurone generation, and that it enhances neurite growth and sustains sympathetic neurone survival at different stages of development.

## MATERIALS AND METHODS

### Neurone culture

Low-density dissociated cultures were established separately from the SCG and from the stellate ganglion together with other ganglia of the thoracic sympathetic chain (SG) of CD1 mouse embryos at 12, 13, 14, 15 and 16 days gestation (E12 to E16), and at birth and postnatal days 4, 8, 12, 16, 20, 35 and 60 (P0 to P60). The dissected ganglia were trypsinised (0.05% trypsin for 15 minutes at 37°C) and dissociated by trituration. P12 and older ganglia were treated with collagenase (0.2% collagenase for 30 minutes at 4°C followed by 20 minutes at 37°C) before trypsinisation. The neurones were grown in defined, serum-free medium on a poly-ornithine/laminin substratum in 35 mm diameter tissue culture petri dishes (Davies et al., 1993). Purified recombinant human artemin or function-blocking mouse monoclonal anti-GFRα3 antibodies were added to the cultures at the time of plating.

To obtain a simple estimate of the number of neurones surviving in these cultures under different experimental conditions, the number of attached neurones within a 12×12 mm grid in the centre of each dish was initially counted 6 hours after plating and was counted again at time intervals thereafter. The number of neurones present in the grid at these later times is expressed as a percentage of the initial count at 6 hours. In each experiment, triplicate cultures were set up for all conditions.

Because neurones are generated from proliferating progenitor cells in cultures of early SCG and SG, the number of neurones surviving in these cultures at intervals following plating is influenced not only by the length of time individual differentiated neurones survive, but also by the rate at which neurones are generated from their progenitors. To quantify both of these parameters in the same experiment, we followed the survival of the neurones that comprised an initial cohort identified shortly after plating, monitored the generation of new neurones at intervals and followed the survival of these newly generated neurones. In these 'cumulative cohort experiments', the initial cohort was identified within a 12×12 mm grid in the centre of 60 mm culture dishes 6 hours after plating. The survival of these neurones was monitored at 6 hourly intervals and is expressed as a percentage of the starting number of neurones in the initial cohort. In addition to following the survival of neurones in the initial cohort, the generation of new neurones in the same grid was monitored at each time point. This established new cohorts of neurones that were generated between 6 and 12 hours, 12 and 18 hours, 18 and 24 hours, 24 and 30 hours, and 30 and 36 hours. The survival of neurones in each of these newly identified cohorts was subsequently monitored at 6 hourly intervals after their identification. The number of neurones in these cohorts is expressed as a percentage of the number of neurones in the initial cohort identified 6 hours after plating. The results of each experiment are plotted in stacking bar charts.

### Neuroblast proliferation in vitro

Neuroblast proliferation was measured in vitro by determining the number of neuroblasts that incorporated bromodeoxyuridine (BrdU) into their nuclei using immunocytochemistry. Cells were plated in 24-well multiwell plates (Costar), BrdU was added at various times after plating the cells and the cultures were incubated for a further period to permit incorporation of BrdU into S-phase cell nuclei. The cells were then fixed in methanol (−20°C for 15 minutes) and were stained for nuclear BrdU incorporation using an anti-BrdU monoclonal antibody (Sigma) diluted 1:500 in phosphate-buffered saline (PBS) for 48 hours at 4°C. The cells were then labelled using biotinylated secondary antibody (1:200), avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was diaminobenzidine tetrachloride (DAB substrate kit, Vector Labs). The number of BrdU-positive cells is expressed as a percentage of total cell number.

### Generation of *GFRα3*$^{-/-}$ mice

A BAC clone containing the *GFRα3* gene was isolated and used to construct a targeting vector. A pGK1-neo cassette flanked 5′ by a 1.6 kb PCR fragment located approximately 1.5 kb upstream of the initiation ATG and 3′ by a 5.3 kb *Eco*RI-*Xho*I fragment immediately downstream of exon 1 was used to delete a 2 kb fragment containing the first exon of GFRα3. Linearised DNA (20 μg) was used to electroporate $1\times10^7$ ES R1 cells. G418/Gancyclovir resistant clones were screened for homologous recombination by Southern analysis. Genomic DNA was digested with *Hin*dIII and hybridised with a 1.5 kb EcoRI-PstI fragment located upstream of the 1.6 kb short arm. Homologous recombination was detected at a frequency of 1/100 clones. Three independent targeted clones were used to generate chimeric animals by injection into C57BL/6 blastocysts. Male chimeras were bred with C57BL/6 females, and heterozygous offspring were interbred to generate *GFRα3*$^{-/-}$ mice. These mice were back-crossed into a CD1 background (the same in which all other experiments were carried out).

### Neuroblast proliferation in GFRα3-deficient embryos

Embryos were obtained from overnight matings of *GFRα3*$^{+/-}$ mice. Pregnant females were killed by cervical dislocation after 14 days gestation, and the genotypes of the embryos were determined by a PCR-based technique using DNA isolated from embryonic bodies. The heads were fixed for 30 minutes in Carnoy's fluid (60% ethanol, 30% chloroform and 10% glacial acetic acid). After dehydration through a graded alcohol series, the tissue was paraffin wax embedded. Serial sections of the heads in the region of the SCG were cut at 8 μm and were mounted onto poly-lysine-coated slides (BDH) or Gold Seal Ultrastick Slides (Erie Scientific).

To monitor neuroblast proliferation, the sections were stained for the presence of the proliferating cell nuclear antigen (PCNA). Because expression of PCNA is not restricted to dividing neuronal cells, the sections were double stained for βIII tubulin to identify all neuroblasts and neurones in the SCG. The sections were cleared in xylene and rehydrated before quenching in 3% hydrogen peroxide in methanol

for 20 minutes. Nonspecific antibody binding was blocked in 10% horse serum, 0.5% Triton X-100 in PBS before incubation with anti-PCNA monoclonal antibody (Sigma) diluted 1:1000 in blocking buffer for 1 hour at room temperature. The cells were then labelled using biotinylated secondary antibody (1:200), avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was 1mg/ml diaminobenzidine tetrachloride (FastDAB, Sigma). The sections were then incubated with mouse anti-βIII tubulin antibody (Promega) diluted 1:5000 in blocking buffer overnight at 4°C. The cells were then labelled using biotinylated secondary antibody (1:200), avidin, biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs) and visualised using a VIP substrate kit (Vector Labs) which produced an intense purple reaction product.

The total number of βIII tubulin-positive cells and the number of βIII tubulin-positive cells that were also PCNA positive were estimated using a digital stereology system that uses a combination of the optical dissector and volume fraction/Cavalieri methods (Kinetics Imaging).

## RESULTS

### Artemin enhances the generation and survival of early sympathetic neurones

We began investigating the role of artemin in sympathetic neurone development by studying its effects on neuronal survival in dissociated cultures established from the SCG and SG at closely staged intervals throughout embryonic development. Preliminary analysis showed that in cultures established throughout the period of neurogenesis from E12 to E15, there were more neurones in artemin-supplemented cultures than in control cultures. Fig. 1 illustrates the dose-dependent nature of this effect of artemin and shows that the most effective concentration for increasing neurone number was 10 ng/ml, which was used in all subsequent experiments.

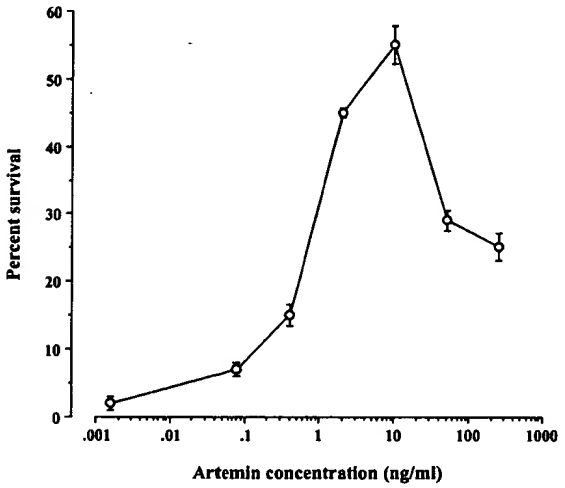


**Fig. 1.** Graph of the number of sympathetic neurones present in E14 dissociated SCG cultures after 48 hours incubation with a range of concentrations of artemin expressed as a percentage of the number of neurones counted 6 hours after plating. The means and standard errors (minus survival in control cultures) of data obtained from three petri dishes for each condition are shown. Similar results were obtained in two separate sets of cultures.

In cultures established after the period of neurogenesis (from E16 to birth), no increase in neurone number was observed in the presence of artemin.

Because sympathetic neurones are generated from proliferating neuroblasts in dissociated cultures of early sympathetic ganglia (Rohrer and Thoenen, 1987), it is possible that artemin could increase the number of surviving neurones in these cultures by either increasing the generation of neurones from neuroblasts or by enhancing neuronal survival. To distinguish between these two alternatives, we set up cumulative cohort experiments. This involved identifying all of the neurones within a grid in the centre of each culture dish 6 hours after plating and following the fate of every neurone in this initial cohort at 6 hourly intervals (i.e. whether it was alive or dead). Differences in rate at which the neurones die between control and artemin-supplemented cultures would indicate whether artemin enhances the survival of differentiated neurones. In addition, all new neurones that were generated in the grid during successive 6 hourly intervals were identified and their fate was likewise followed over time. These latter data would indicate whether artemin influences the generation of neurones from neuroblasts. Fig. 2 shows the results of typical cumulative cohort experiments set up at daily intervals from E12 to E16. In all cultures, the survival of the initial cohort of neurones was enhanced in the presence of artemin compared with control cultures. This survival-promoting effect of artemin was most marked in E12 to E14 cultures, it was less evident in E15 cultures and was negligible in E16 cultures. These results indicate that artemin has a direct survival-enhancing action on neurones present in the early sympathetic chain, but that this effect is transient and not maintained beyond E16.

The cumulative cohort experiments showed that new neurones are generated in early sympathetic chain cultures. Most new neurones were generated in E12 cultures and the number of new neurones decreased with age, becoming negligible by E16 (Fig. 2). Expressed as a percentage of the size of the initial 6 hour cohorts, the total number of new neurones generated in control cultures between 6 and 36 hours decreased from 70% in E12 cultures to 3% in E16 cultures (Fig. 3). At all ages, more neurones were generated in artemin-supplemented cultures, the increase was most pronounced in E12, E13 and E14 cultures, when neurogenesis is greatest in control cultures. In E12 to E14 cultures there were 60% more new neurones generated in the presence of artemin (Fig. 3). This increase in neurogenesis in the presence of artemin was evident during each 6 hourly interval in culture (Fig. 2). The results of these cumulative cohort experiments suggest that artemin increases the proliferation of sympathetic neuroblasts.

To provide an additional measure of neuroblast proliferation, we studied BrdU incorporation in dissociated cultures established from E12 and E13 SCG and SC, stages when neurogenesis is highest. Artemin increased the number of BrdU-positive neurones in E12 and E13 cultures by 76% and 55%, respectively (Fig. 3). These results clearly demonstrate that artemin promotes sympathetic neuroblast proliferation in vitro.

### Reduced neuroblast proliferation in GFRα3-deficient embryos

To ascertain whether the effects of artemin on neuroblast proliferation observed in vitro are physiologically relevant, we

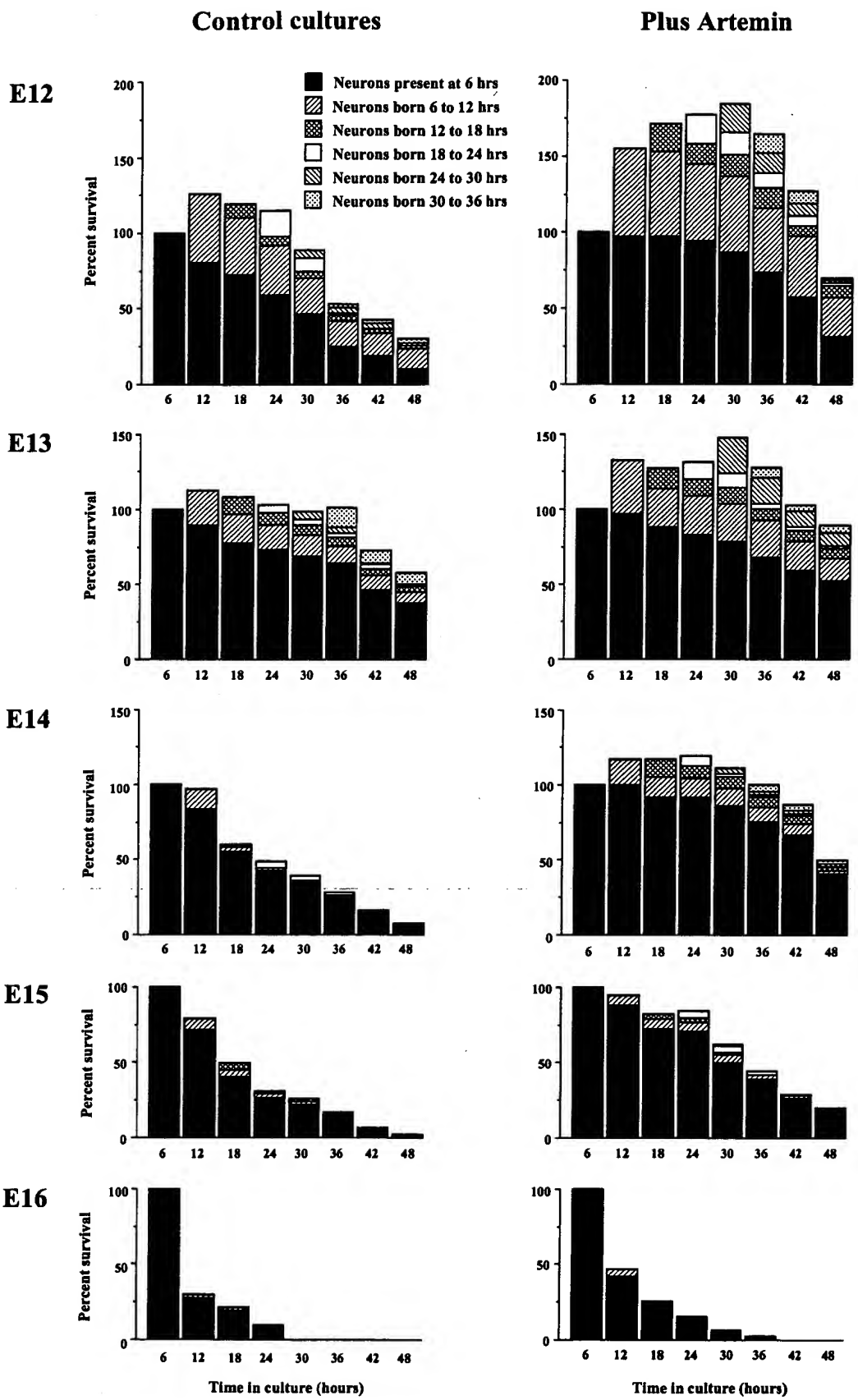


**Fig. 2.** Stacking bar charts of representative cumulative cohort experiments set up from the SCG and SG of E12 to E16 embryos (SCG at E12, E14 and E16, and SG at E13 and E15). The cells were grown in either defined medium alone (control cultures) or medium supplemented with 10 ng/ml artemin. An initial cohort comprising all the neurones in a 12×12 mm grid in the centre of each dish was identified 6 hours after plating. The number of surviving neurones in this cohort was monitored at 6 hourly intervals and is expressed as a percentage of the initial cohort at 6 hours (black bars). New neurones that were generated in the grid between 6 and 12 hours, 12 and 18 hours, 18 and 24 hours, 24 and 30 hours, 30 and 36 hours are expressed as a percentage of the initial cohort, and their survival was likewise subsequently monitored at 6 hourly intervals after their appearance (patterned and white bars).

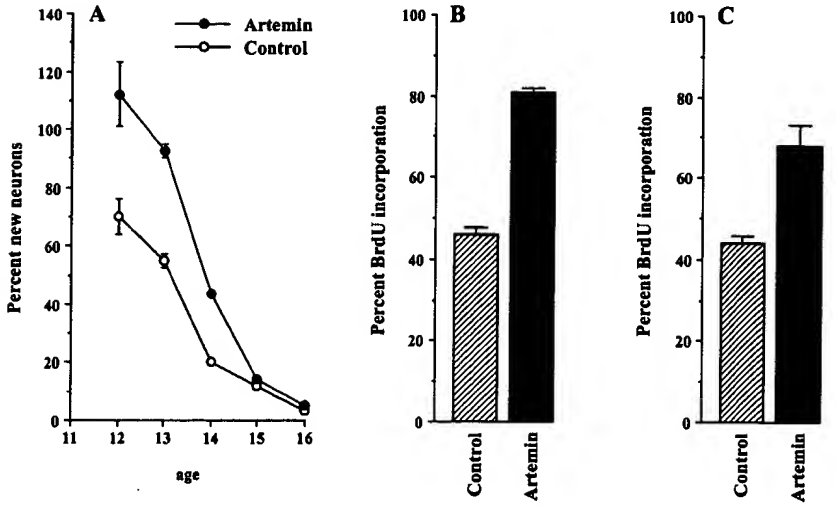


**Fig. 3.** (A) Graph of the number of new neurones generated between 6 and 36 hours after plating in dissociated cultures of sympathetic ganglia of E12 to E16 embryos in defined medium alone (control) or medium supplemented with 10 ng/ml artemin. Bar charts of the number of BrdU-labelled neurones in dissociated E12 SCG (B) and E13 (C) stellate ganglion cultures grown either in defined medium alone (control) or medium supplemented with 10 ng/ml artemin. In E12 cultures, BrdU was added 2 hours after plating and the cultures were fixed and stained 16 hours after plating. In E13 cultures, BrdU was added 24 hours after plating and the cultures were fixed and stained 12 hours later. The means and standard errors are shown ($n$=3-6 for each condition).

generated GFRα3-deficient mice by gene targeting and compared neuroblast proliferation in the E14 SCG of wild-type and *GFRα3*$^{-/-}$ embryos. A targeting vector where the first exon containing the signal sequence was replaced by a PGK1-neo cassette was transfected into embryonic stem cells (Fig. 4). Gene targeting was detected in 1/100 ES colonies and three clones were selected for microinjection into blastocysts. Chimeric mice with appropriate germline transmission were back-crossed into a CD1 background.

For the experimental studies, *GFRα3*$^{+/-}$ mice were crossed and pregnant females were killed after 14 days gestation. Wild-type and *GFRα3*$^{-/-}$ embryos were fixed, paraffin wax embedded and serially sectioned through the SCG. Proliferating cells were detected by staining these sections with an antibody against proliferating cell nuclear antigen (PCNA), a protein that is expressed at high levels in the nuclei of S-phase cells (Kurki et al., 1986). Although the great majority of cells in E14 SCG are neuroblasts or post-mitotic neurones, to ensure that only PCNA-positive neuroblasts were counted, we also stained these sections for β-III tubulin, which labels neuroblasts and post-mitotic neurones. Confirming the results of Nishino and colleagues (Nishino et al., 1999), we found that the SCG of *GFRα3*$^{-/-}$ embryos were smaller and more caudally located than the SCG of wild-type embryos. This difference in the overall size of the SCG of *GFRα3*$^{-/-}$ embryos was associated with a reduction in the number of β-III tubulin-positive cells compared with wild-type embryos in the same litter (Fig. 5). Importantly, the proportion of β-III tubulin-positive cells that were PCNA-positive in the SCG of *GFRα3*$^{-/-}$ embryos was half that observed in wild-type embryos. These results indicate that a smaller proportion of neuroblasts undergo mitosis in the SCG of GFRα3-deficient embryos compared with wild-type

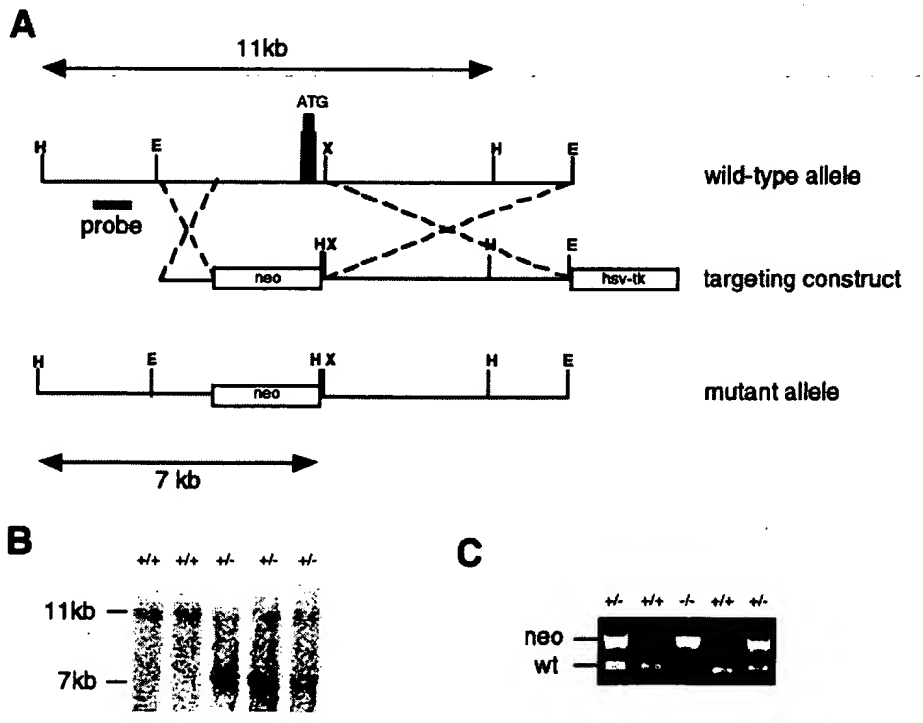


**Fig. 4.** Generation of GFRα3 knockout mice by homologous recombination. (A) Gene targeting vector. A Neo cassette replaced a fragment containing exon one, deleting the start codon and the signal sequence of GFRα3. H, *Hin*dIII; E, *Eco*RI; X, *Xho*I. (B) Southern blot screening of targeted ES clones. Genomic DNA was digested with *Hin*dIII. The probe, upstream of the short arm (shown in A), detected the 11 kb wild type and 7 kb mutant fragments. (C) PCR genotyping of tail DNA prepared from offspring of intercrossing heterozygous mice. Tail DNA was amplified with a primer set specific for the Neo gene, which detects the mutant allele, and a primer set that hybridises within the sequence that is deleted in the mutant and therefore detects the wild-type allele.

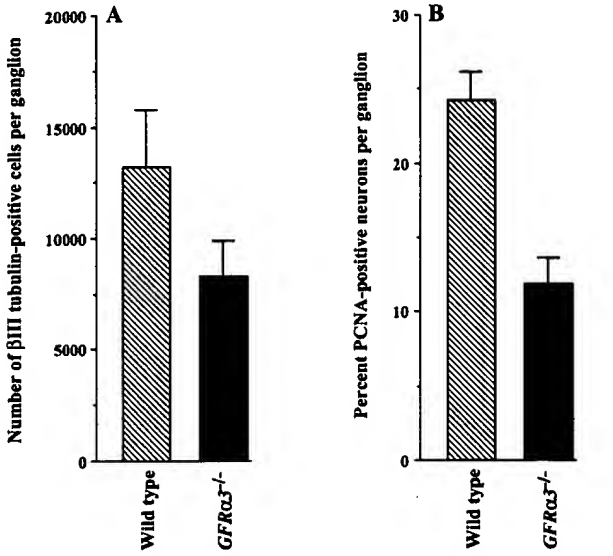


**Fig. 5.** Bar charts of (A) the total number of βIII-tubulin-positive and (B) the percent of βIII-tubulin-positive cells that are PCNA positive in E14 wild-type and *GRFα3*$^{-/-}$ embryos. The means and standard errors are shown (data derived from six wild-type and five *GRFα3*$^{-/-}$ embryos).

embryos, suggesting that the GFRα3 ligand artemin promotes sympathetic neuroblast proliferation in vivo.

## Artemin has a transient survival effect on mature SCG neurones

To investigate whether artemin affects the development of sympathetic neurones after birth and in the adult, we investigated the effects of artemin on SCG neurones in low-density dissociated cultures established at intervals throughout the postnatal period and into adulthood. From birth to P8, neurones in artemin-supplemented cultures died as rapidly as neurones in control cultures, so that by 48 hours incubation all neurones grown in control and artemin-supplemented cultures had died. By contrast, nerve growth factor (NGF) promoted the survival of the majority of these neurones and survival was neither enhanced nor reduced by the concomitant presence of artemin (Fig. 6).

As sympathetic neurones began to lose their survival dependence on NGF with age, they started to exhibit a clear survival response to artemin. In P12 cultures, the neurones died much more slowly in control cultures than at earlier postnatal stages, so that by 48 hours incubation, 30% of neurones were still surviving in defined medium alone. In artemin-supplemented cultures at this age, the neurones died more slowly than in control cultures, so that by 48 hours there were 66% neurones still surviving in the presence of artemin. From P16 to P60, the neurones survived increasingly well in the control cultures, so that after 48 hours incubation in P60 cultures, more than half of the neurones were still surviving without neurotrophic factors. Throughout this period, the neurones survived increasingly well with artemin, so that by P20 the neurones survived as well in artemin-supplemented cultures as in cultures containing NGF. There were no significant differences in the number of neurones surviving with NGF alone or NGF plus artemin at all ages studied, indicating the artemin-responsive neurones comprise a subset of NGF-responsive neurones. The changes in the response of neurones to artemin in the postnatal period in relation to their survival under other conditions are summarised in Fig. 7. These results show that sympathetic neurones acquire a late survival response to artemin during the postnatal period.

The appearance of a survival response to artemin just before sympathetic neurones begin to lose dependence on neurotrophic factors for survival raised the possibility that endogenously produced artemin might account for the ability of adult sympathetic neurones to survive without added neurotrophic factors. Neurotrophic factor autocrine loops have previously been described for BDNF in neurotrophic factor independent embryonic and adult DRG neurones (Wright et al., 1992; Acheson et al., 1995) and for HGF in sympathetic neuroblasts (Maina et al., 1998). To investigate the possibility that artemin released into the culture medium of adult sympathetic neurones is responsible for sustaining the survival of these neurones in the absence of added neurotrophic factors, we used a function-blocking anti-GFRα3 monoclonal antibody. In initial experiments, we confirmed that this antibody inhibited the survival enhancing effects of artemin, and by using different concentrations of this antibody we determined that 100 ng/ml of this antibody was sufficient to block completely the survival-enhancing effects of 10 ng/ml artemin in cultures of E14 SCG neurones (data not shown). Fig. 8 shows that this concentration of antibody also completely inhibited the survival-enhancing effect of artemin on P60 SCG neurones but did not affect the response of the neurones to NGF. The anti-GFRα3 antibody did not reduce the survival of P60 SCG neurones grown without added neurotrophic factors in the culture medium, indicating that the survival of these adult neurones in the absence of added neurotrophic factors is not dependent on the artemin synthesised in these cultures acting on GFRα3 receptors. We also investigated whether endogenously produced artemin plays a role in sustaining sympathetic neurones during the early phase of neurotrophic factor independence. In E14 cultures the anti-GFRα3 antibody did not reduce neuronal survival in the absence of added neurotrophic factors, indicating that endogenously produced artemin released from cells in these cultures does not play a role in sustaining the generation or survival of early sympathetic neurones.

## Artemin enhances the growth of SCG neurone arbours

To investigate the potential effects of artemin on sympathetic neurite growth, it was essential to compare the same subset of neurones grown with and without artemin. Because more neurones survive in artemin-supplemented cultures than in control cultures, even at P60 (when many neurones survive without added neurotrophic factors), simply comparing neurite growth in artemin-supplemented and control cultures would not distinguish between direct neurite-growth promoting effects of artemin and neurite growth that was secondary to the enhanced survival and well being of the neurones in the presence of artemin. However, our finding that the number of neurones surviving in cultures containing NGF plus artemin was no greater than the number surviving in cultures containing NGF alone enabled us to investigate the effects of artemin on

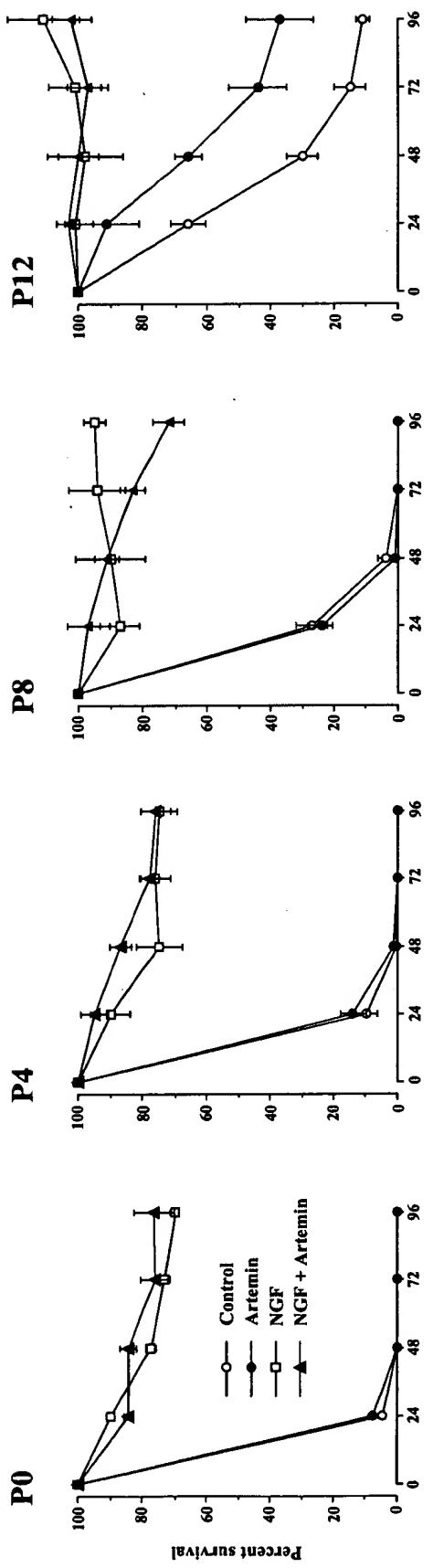


**Fig. 6.** Graphs of the percent survival of P0 to P60 SCG neurones grown in either defined medium alone (control) or in medium supplemented with 10 ng/ml artemin, 10 ng/ml NGF or artemin plus NGF. The number of neurones surviving 24, 48, 72 and 92 hours after plating is expressed as a percentage of the number of neurones counted 3 to 6 hours after plating. The means and standard errors are shown ($n$=6-9 for each condition).

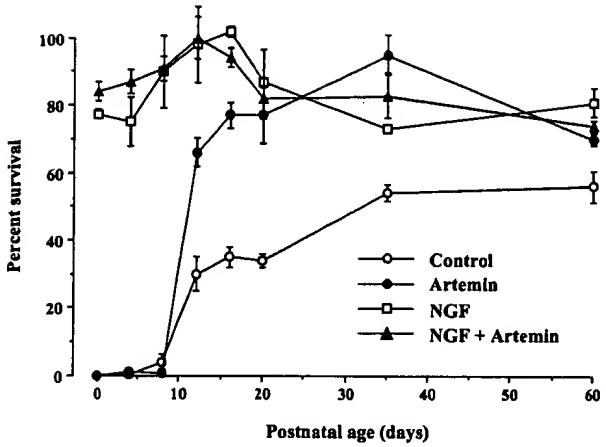


**Fig. 7.** Graph summarising the developmental changes in the percent survival of SCG neurones grown in either defined medium alone (control) or in medium supplemented with 10 ng/ml artemin, 10 ng/ml NGF or artemin plus NGF in cultures established from P0 to P60 mice. The means and standard errors are shown ($n$=6-9 for each condition).

neurite growth independently of survival, and to determine whether artemin was able to enhance neurite growth beyond that observed with NGF alone. Neurones were grown in medium containing NGF in the presence or absence of artemin and the total length of the neurite arbours was estimated. Cultures were set up at stages throughout sympathetic neurone development. At stages between E14 and P3, there were no significant differences in the length of the neurite arbours in cultures containing NGF alone or NGF plus artemin (Fig. 9). However, at P20 (after the neurones have acquired a survival responsive to artemin), the length of neurite arbours was 47% longer in the presence of NGF plus artemin compared with NGF alone. Likewise, at P60, artemin promoted a similar increase in neurite length. These results show that in the late postnatal period, artemin increases neurite growth beyond that occurring in the presence of NGF alone.

## DISCUSSION

To clarify the role of artemin in sympathetic neurone development, we have undertaken a detailed in vitro analysis of its effects on sympathetic neurone generation, survival and growth in dissociated cultures established from sympathetic ganglia at stages throughout development, and complimented these in vitro studies with in vivo analysis of neuroblast proliferation in GFRα3-deficient embryos. Our findings have shown that artemin has several distinct effects at different stages of development. First, it enhances sympathetic neuroblast proliferation and increases the generation of sympathetic neurones. Second, it enhances the survival of sympathetic neurones at two distinct stages in their development: initially for a brief period after they have undergone their terminal mitosis in the embryo and once again in the late postnatal period, shortly before they acquire neurotrophic factor-independent survival. Third, it enhances neurite growth from late postnatal and adult sympathetic neurones.

The effect of artemin on enhancing neuroblast proliferation and the generation of post-mitotic sympathetic neurones was revealed by studying BrdU incorporation in dissociated cultures established from early sympathetic ganglia, and by detailed analysis of the generation of new neurones in cumulative cohort experiments set up throughout the phase of neurogenesis in the sympathetic chain. The physiological relevance of these in vitro observations was substantiated by our demonstration that there is a marked reduction in the proportion of sympathetic neuroblasts in the mitotic phase of the cell cycle in embryos deficient in the GFRα3 subunit of the artemin receptor. Although a reduction in the size of the SCG has been reported in GFRα3-deficient embryos, this was attributed to a failure of SCG precursors to complete the last step in their rostral migration from lower cervical levels (Nishino et al., 1999). Although this is the likely reason for the more caudal location of the SCG in

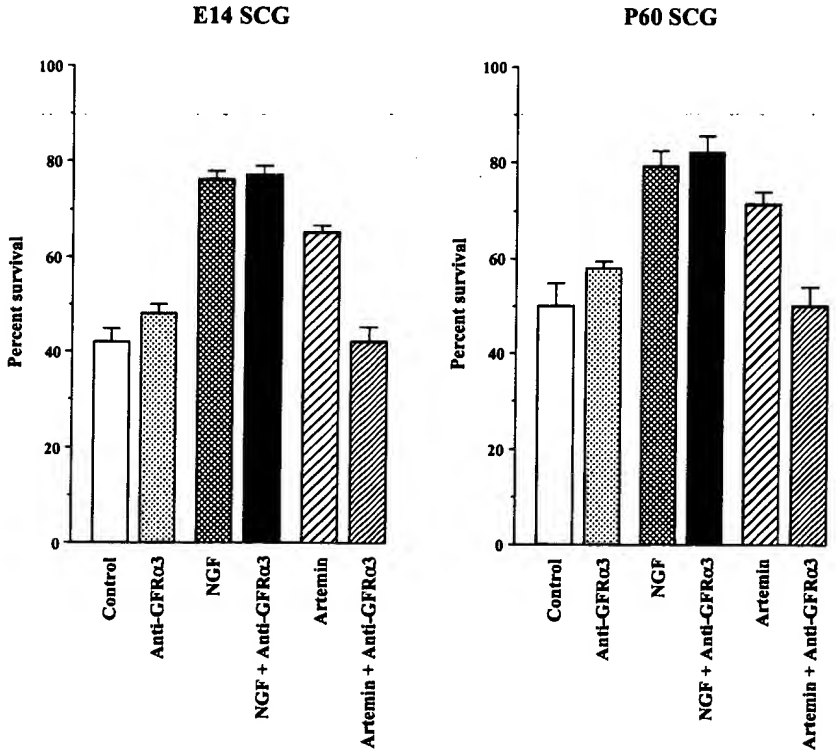


**Fig. 8.** Bar charts of the numbers of surviving neurones in cultures of E14 and P60 SCG grown for 48 and 72 hours, respectively, in either defined medium alone (control) or in medium supplemented with 100 ng/ml anti-GFRα3 antibody, 10 ng/ml NGF, 10 ng/ml artemin, anti-GFRα3 plus NGF or anti-GFRα3 plus artemin. The means and standard errors are shown ($n$=6-9 for each condition).

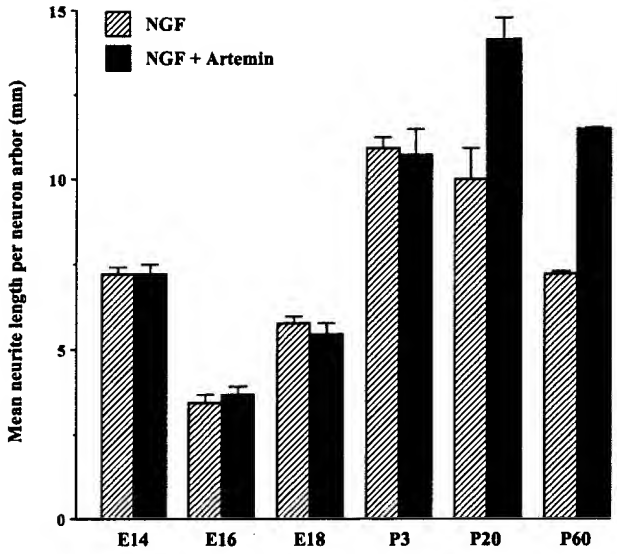


**Fig. 9.** Bar charts of the total length of the neurite arbours growing from SCG neurones in cultures established from E14 to P60 animals after 48 hours incubation with NGF alone or NGF plus artemin (both at 10 ng/ml). The means and standard errors are shown ($n$=4-9 for each condition).

*GFRα3*$^{-/-}$ embryos, and may contribute to the reduced size of this ganglion in these embryos, our findings suggest that a major contributing factor to the decreased size of the SCG is a marked reduction in neuroblast proliferation. A local action of artemin on neuroblast proliferation within the early SCG is consistent with the in vivo expression of artemin and GFRα3. By in situ hybridisation, artemin mRNA is detectable in the SCG and in the region surrounding the SCG precursors, and GFRα3 mRNA is expressed by the majority of cells in the SCG during this early stage in development (Nishino et al., 1999).

What little is known about the signals that potentially regulate sympathetic neuroblast proliferation has been gleaned from in vitro studies of these cells obtained from mammalian and avian embryos. It has been reported that insulin, insulin-like growth factor 1, pituitary adenylate cyclase-activating polypeptide (PACAP), NT3 and retinoic acid promote the proliferation of rat sympathetic neuroblasts in culture (DiCicco-Bloom and Black, 1988; DiCicco-Bloom and Black, 1989; DiCicco-Bloom et al., 1993; Wyatt et al., 1999; DiCicco-Bloom et al., 2000). However, the physiological significance of these in vitro observations is unclear. In the case of NT3, appropriate numbers of sympathetic neurones are generated in NT3-deficient mouse embryos, and enhanced neuronal death occurs only after the phase of neurogenesis is over (Wyatt et al., 1997; Francis et al., 1999). Likewise, the demonstration that CNTF decreases neuroblast proliferation in cultures of embryonic chicken sympathetic chain (Ernsberger et al., 1989) is unclear because in rodents, at least, CNTF synthesis does not commence until after birth (Dobrea et al., 1992). By using complimentary in vitro and in vivo approaches, we have provided the first clear evidence that an identified growth factor plays a physiologically significant role in promoting the proliferation of sympathetic neuroblasts, leading to the increased generation of sympathetic neurones.

Analysis of neuronal survival in cohort experiments established from early sympathetic ganglia clearly shows that artemin sustains the survival of newly generated sympathetic neurones for a brief period after they have undergone their terminal mitosis. This survival effect is no longer evident after E16, and no survival effect of artemin was observed on SCG neurones cultured between this stage and P8. However, by P12, a marked survival response to artemin was observed which was sustained through later ages. The appearance of this late survival response to artemin coincides with the ability of an increasing proportion of sympathetic neurones to survive in culture without added neurotrophic factors. However, the possibility that the production and release of artemin within cultures of mature sympathetic neurones is responsible for the appearance of neurotrophic factor-independent survival is excluded by the lack of effect of function-blocking anti-GFRα3 antibodies on the survival of mature sympathetic neurones.

Previous in vitro studies have reported that artemin promotes the survival of newborn rat sympathetic and sensory neurones (Baloh et al., 1998), and postnatal mouse sensory neurones (Baudet et al., 2000). However, our detailed developmental study has revealed a novel phenomenon that has not previously been described for any other neurotrophic factor, namely, that the same population of neurones exhibits two distinct phases of responsiveness to a factor separated by an extended period during which the factor has no effect on survival. These two phases in the survival-promoting action of artemin on embryonic and late postnatal sympathetic neurones observed in vitro might correspond to local and target-derived actions of artemin, respectively, on sympathetic neurone survival in vivo.

Curiously, although artemin did not promote the survival of cultured SCG neurones between birth and P8 (Figs 6 and 7), these neurones start to die at an abnormally high rate in GFRα3-deficient mice during this period of development (Nishino et al., 1999). It is possible that this abnormal death of SCG neurones may be because absence of artemin/GFRα3 signalling earlier in development might have affected the ability of the neurones to innervate their targets appropriately and to obtain an adequately supply of target-derived NGF and NT3 on which they depend for their survival (Levi-Montalcini and Booker, 1960; Zhou and Rush, 1995). It should be noted that the SCG of *GFRα3*$^{-/-}$ mice forms in an abnormal anatomical location (Nishino et al., 1999) that might affect the subsequent guidance of sympathetic axons to their correct targets.

In addition to enhancing the survival of late postnatal sympathetic neurones, artemin increased neurite growth from these neurones to a greater extent than NGF. The effects of NGF on promoting neurite growth are well documented both in vitro and in vivo (Edwards et al., 1989; Purves et al., 1988; Scott and Davies, 1993; Snider, 1988). Recently, other neurotrophic factors have been shown to synergise with NGF in promoting neurite outgrowth. For example, HGF enhances neurite growth from embryonic sensory and sympathetic neurones in the presence of NGF (Maina et al., 1998; Maina et al., 1997) and enhances neurite growth from postnatal SCG neurones (Yang et al., 1998). It is possible that the effect of artemin on neurite growth observed in vitro may reflect a role for artemin in establishing and maintaining target field innervation in vivo. Indeed, the loss of this effect of artemin in

GFRα3-deficient mice may at least be partly responsible for the observed deficiency of sympathetic axons in several tissues in the postnatal period (Nishino et al., 1999).

In summary, we have defined the roles of artemin at different stages in the development of the sympathetic nervous system by studying its effects on neuroblasts and neurones in culture and by analysing embryos defective in artemin signalling. We have shown that artemin increases sympathetic neuroblast division, and provide the first evidence that a growth factor is a physiologically relevant regulator of the proliferation of these neuroblasts. Artemin subsequently promotes the survival of newly differentiated sympathetic neurones for a brief period after they have undergone their terminal mitosis. Then, uniquely for a neurotrophic factor, artemin promotes the survival of these neurones for a second time several weeks after birth. During this late stage in their development, artemin also enhances neurite growth to a greater extent than NGF. Our results provide a clear illustration of how a single factor can exert a diversity of actions on a population of neurones at different stages in its development and raise interesting questions about the nature of the signalling pathways mediating these different responses.

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# Agonist antibody and Fas ligand mediate different sensitivity to death in the signaling pathways of Fas and cytoplasmic mutants

We have produced three forms of human Fas: full-length Fas, Fas with a C-terminal deletion, and a chimera between extracellular Fas and the intracellular domain of the tumor necrosis factor receptor I p55 subunit. We transfected cell lines with these constructs to compare the relative capacity of antibody agonists and the physiological Fas ligand (FasL) to stimulate death. With two agonistic antibodies, the chimera is 100- to 1000-fold more sensitive to induction of death than the full-length Fas. The C-terminal deletion mutant also shows greatly enhanced death in comparison to the wild-type receptor. In contrast, when FasL is used to trigger the Fas pathway, wild-type Fas and the deletion mutant are similarly sensitive, whereas the chimera is 100-fold less susceptible to ligand-mediated killing than Fas. This demonstrates that antibody agonists and natural ligand can stimulate different signaling pathways and emphasizes the limitations of defining physiologically important signaling pathways solely by antibody agonists.

## 1 Introduction

Fas (Apo-1/CD95) is a cell surface protein that induces apoptosis. It is a member of the tumor necrosis factor receptor (TNFR) superfamily and was originally characterized by antibodies against tumor cells [1, 2]. The *lpr* mouse has a recessive mutation that results in a defect in the expression of Fas. In this mouse, a 5.4-kb transposon has been inserted into the second intron of the Fas gene, resulting in premature termination and faulty splicing of the Fas mRNA [3]. Mice with the *lpr* mutation develop severe lymphadenopathy, glomerulonephritis, autoantibody production, immune complex disease, and hypergammaglobulinemia. Fas is a member of the TNFR family that shares homologous regions not only in the extracellular, but also in the intracellular domain of TNFRI (p55). The death domain is a 45–80 amino acid region (depending on the alignment used) shared by Fas and TNFRI with 51 % homology between the two proteins including conservative nucleotide changes [4, 5]. Considerable effort has been devoted to finding similarities and differences between these two signaling pathways.

In this report, we compare various forms of Fas and their ability to mediate apoptosis in different assays. Three forms of human Fas were produced; full-length Fas, Fas with a 15-amino acid C-terminal deletion (FD5) reported to enhance activity [6], and a chimera between extracellular human Fas and intracellular mouse TNFRI. Using antibodies against Fas, we confirmed the observation that the deletion mutant is more sensitive to mediate death than full-length Fas. The chimeric version of Fas (Fas-TNFRI) is equally or more sensitive to anti-Fas antibody than the deletion mutant. Surprisingly, this order of potency could not be reproduced when Fas ligand (FasL) was used as the effector molecule. When activated T cells expressing FasL, L cells transfected with FasL or soluble ligand were used as effectors, targets expressing full-length Fas or the deletion mutant exhibited similar sensitivities. The chimeric protein had a dramatically reduced capacity to signal death in response to FasL. These findings demonstrate a clear difference between antibody versus ligand in stimulating the Fas-mediated death pathway.

## 2 Materials and methods

### 2.1 Mice

C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6Smn.C3H-*FasL*$^{gld}$ (B6.*gld*) mice were bred in our own facility, originating from breeding pairs purchased from Jackson Laboratories.

### 2.2 Cells

EL4 cells were cultured in T5 (RPMI 1640 supplemented with glutamine, mercaptoethanol, nonessential amino acids, Hepes, and heat-inactivated newborn calf serum). COS cells were cultured in D10 (DMEM supplemented with glutamine, mercaptoethanol, Hepes and heat-inactivated fetal calf serum). B6 effector cells were prepared by mixed lymphocyte reaction. Briefly, $5 \times 10^6$ irradiated BALB/c (H-2$^d$) spleen cells were plated out with $1 \times 10^6$ C57BL/6 (H-2$^b$) spleen cells and 10 U/ml mouse IL-2/1.5 ml K5 (T5 with fetal calf serum rather than newborn calf serum) [7]. Cells were then stimulated once per week and used 3–5 days after stimulation. Lines maintained in this fashion are >95 % CD8$^+$ (data not shown).

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**Abbreviations:** **FasL:** Fas ligand **TNFRI:** Tumor necrosis factor receptor I (p55)

**Key words:** Fas / Apoptosis / Programmed cell death / CD95

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### 2.3 PCR

PCR was performed using Perkin Elmer AmpliTaq (Foster City, CA). Buffer conditions were determined using the Opti-Prime PCR Optimization Kit (Strategene, La Jolla, CA). Cycling conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min for 35 cycles. All constructs were sequenced to assure fidelity of the PCR products. Human full-length Fas was cloned from E6 cells (Jurkat) by reverse transcription (RT)-PCR using 5′ primer: GAATTCAAGCTTGTTGGGGAAGCTCTTTCACT (154–173) and 3′ primer: GAATTCAAGCTTCTAGACCAAACTCTGGATTTCATTTCTGAAG (1172–1204). The Fas deletion mutant was produced using the Fas plasmid as a template. The same 5′ forward primer used to clone full-length Fas and a 3′ reverse primer with the 15 terminal amino acids deleted and containing a stop codon [GAATTCAAGCTTCTAACTAGTAATGTCCTTGA (1138–1154)] were used in a PCR reaction. TNFRI was produced using the 5′ primer: GAATTCAAGCTTAATTCTCTGGACTGAGGCTC (63–79) and 3′ primer: GAATTCAAGCTTAGGACCCCTCCTTTCCAGAA (1473–1490). The Fas-TNRFI chimera was produced by a two-step PCR method. Mouse TNFRI was cloned from L929 cells by RT-PCR. In the first round of PCR, a plasmid containing human Fas was used as a template. The same 5′ forward primer specific for Fas and the reverse chimeric 3′ primer: CACCGCAGTACCTGA/TTCCTCTTTGCACTTGGTGT (720–734 TNFR/679–698 Fas) were used to produce a 500-bp fragment encoding the extracellular region of Fas and 15 base pairs of TNFRI. The chimeric PCR product, a plasmid containing mouse TNFRI, a 5′ Fas primer, and a 3′ TNFRI primer were used in the next round of PCR.

The chimeric product contains the extracellular domain of Fas (amino acids 1–152 starting with the first amino acid of the mature Fas protein) and the transmembrane and intracellular domains of TNFRI (amino 179–425). The TNFR 3′ primer was GAATTCAAGCTTAGGACCCCTCCTTTCCAGAA (1473–1490). The primers used to screen transfected cells were: 5′ primer TCAAGGAATGCACAC (655–669), 5′ primer AACCAAGGTTCTCATG (810–825), and 3′ primer from the vector: TTATTCAGGCGTAGCAACCA.

### 2.4 Transfection

EL4 cells were transfected by one of two methods. Cells ($2 \times 10^7$) were electroporated with 10–15 μg plasmid DNA using the BTX Transfector 300 Electroporation System (Biotechnologies & Experimental Research Inc.). Cells were washed twice in PBS and resuspended in cold PBS supplemented with 15 mM Hepes and 0.02% glucose and incubated with plasmid DNA on ice for 5 min. The mixture was then transferred to an electroporation chamber with a 4-mm gap. Electroporation was performed at 450 mV; 250 μF for a pulse length of 20 ms. After pulsing, the chamber was incubated on ice for 5 min and the cells were resuspended in warm T5 at $5 \times 10^5$ cells/ml and incubated overnight at 37 °C. Live cells were harvested on a discontinuous density gradient of 1077 Ficoll Hypaque (Sigma, St. Louis, MO) and replated at $3 \times 10^5$ cell/ml. After 48 h, cells were given 50 μg/ml geneticin (G-418 sulfate; Gibco BRL).

Alternatively, the N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) transfection-reagent (Boehringer Mannheim) was used. Cells were grown to $10^5$–$10^6$/ml in T5. DNA (5 μg) was diluted to a concentration of 0.1 μg/μl in 20 mM Hepes pH 7.4 into a final volume of 50 μl. DOTAP (30 μl) was diluted into 100 μl with Hepes-buffered saline (HBS; 20 mM Hepes, 150 mM NaCl pH 7.4). The DNA and DOTAP were mixed together and incubated at room temperature for 10 min. Cells were added to the mixture in 6 ml T5 medium. After a 3–6-h incubation at 37 °C, the cells were washed two or three times with PBS and incubated in fresh medium for 48–72 h, after which selection medium containing G418 was added. At least two independent transfectants were tested for each construct.

COS cells were transiently transfected with a construct containing the extracellular domain of murine FasL kindly provided by S. Nagata [8]. Cells were plated between 7.5 $\times 10^5$ – $1 \times 10^6$ cells/10-cm plate 24 h prior to transfection. Cells were washed in PBS and resuspended in 4 ml D10. DNA (5 μg) were precipitated and resuspended in 40 μl Tris-buffered saline (TBS). The DNA was then added to 80 μl warm DEAE-dextran (10 μl/ml) in TBS. This mixture was added to the COS cells by swirling the plate. After a 4-h incubation at 37°C, the DNA/media mixture was aspirated; 5 ml of 10% DMSO was then added for 60 s and aspirated. Subsequently, 10 ml K10 was added and cells were maintained at 37°C. After 24 h cells were washed with PBS and 10 ml of 1% fetal calf serum in DMEM was added. Cells were incubated at 37°C for 72 h. Supernatant was harvested and spun for 5 min 2000 rpm to remove any dead cells. Lastly the supernatant was concentrated with Millipore 10000 MW filter and used in killing assays.

### 2.5 FACS staining

Cells were stained with either 7C11 generously provided by Dr. Jerome Ritz [9] or with CH11 purchased from Upstate Biotechnology (Lake Placid, NY) and Medical and Biological Laboratories Co., LTD, Japan. Analyses were performed on a Becton Dickinson FACS System using Cell Quest software (Becton Dickinson, Sunnyvale, CA). Goat anti-mouse Ig conjugated with fluorescein secondary reagents were purchased from Southern Biotechnology (Birmingham, AL).

### 2.6 Antibody-mediated killing assays

Targets were incubated with 100 μCi $^{51}Cr$ (NEN Research Products, Boston, MA) for 2 h. Cells were washed three times with T5, plated at $3 \times 10^4$ cells/well with 7C11 antibody (anti-Fas) in 100 μl, and incubated for 24 h at 37 °C. Alternatively, cells were incubated with 7C11 on ice for 30 min and washed three times. They were plated at $3 \times 10^4$ cells/well with 50 μg/ml goat anti-mouse Ig conjugated with fluorescein from Southern Biotechnology in a final volume of 100 μl, and incubated for 24 h at 37 °C. Supernatants were collected using harvesting frames from Skatron (Lier, Norway) and counted on a Beckman 4000 gamma counter (Carlsbad, CA).

### 2.7 Ligand-mediated killing assays

Target cells were labeled with $^{51}Cr$ as above. Target cells ($1 \times 10^4$) were plated with T cells or mouse FasL-expressing L cells (generously provided by Amy M. Rogers) in 100 µl at different effector-to-target (E:T) ratios for 6 h at 37°C. Effector cells were activated with PMA (Sigma) or anti-CD3 antibody. Effectors were pretreated with 50 µM PMA for 30 min and washed three times before incubating with the target cells. Alternatively, PMA was added to the target and effector cells for the duration of the assay at a final concentration of 50 µM. Various concentrations of anti-CD3 antibody were used to coat 96-well plates (Immulon 3 Dynatech Laboratories, Chantilly, VA) and assays were done at a 30:1 E:T ratio. Supernatants were collected using harvesting frames from Skatron and counted on a Beckman 4000 gamma counter.

Soluble ligand-mediated killing assays were performed by labeling target cells with $^{51}Cr$ as above. Target cells ($1 \times 10^4$) were plated with various dilutions of soluble ligand for 6 h at 37°C. Supernatants were collected using harvesting frames from Skatron and counted on a Beckman 4000 gamma counter.

## 3 Results

### 3.1 Transfected EL4 cells express Fas and Fas variants at equivalent levels

Human Fas was cloned from E6 cells (Jurkat) by RT-PCR and ligated into a vector with a splenic foci forming virus (SFFV) promoter to drive Fas expression and an SV40 promoter for the neomycin resistance gene. This construct was then transfected into EL4 cells (mouse T lymphoma). The EL4 cell line was selected because it expresses low levels of endogenous mouse Fas and is resistant to Fas-dependent killing. After selection with G418, the transfected cells were screened for Fas expression (Fig. 1a).

Two Fas variants were produced by RT-PCR. The FD5 mutant first described by Itoh and Nagata [6] was produced by eliminating the terminal 15 amino acids of Fas. It was previously shown that this deletion mutant enhances the ability of anti-Fas antibody to kill a fibroblast line transfected with the mutant, suggesting that this region functions as an inhibitory domain via interaction with regulatory proteins. The second variant is a chimera between human Fas and mouse TNFRI (p55). This reaction produced a 1.3-kb chimeric fragment with the extracellular domain of human Fas and the transmembrane and intracellular domains of mouse TNFRI. TNFRI and Fas have been implicated on the basis of a number of lines of evidence to have similar signaling pathways that induce death. The most convincing piece of data is the sequence homology between Fas and TNFRI in the intracellular death domain [5]. Tartaglia et al. [5] demonstrated that some TNFR signaling function was retained when the death domain of Fas replaced the death domain of TNFRI. It has also been predicted that like TNFR, Fas must trimerize to expose a chain motif or create a tertiary structure for signaling [10]. Both the deletion mutant and the chimera were sequenced and transfected into EL4 cells which were screened by FACS for expression. As shown in Fig. 1a, the expression levels are nearly equivalent for all three constructs. RT-PCR analysis of the transfected cell lines verified that they encoded the appropriate receptor (Fig. 1b).

### 3.2 Fas and Fas variants are susceptible to anti-Fas antibody-mediated killing

Many earlier studies have focussed on the effect of antibodies on various cell lines that express both Fas and TNFRI. It was shown that sensitivity to antibody-mediated killing does not correlate with expression levels, *i.e.* some

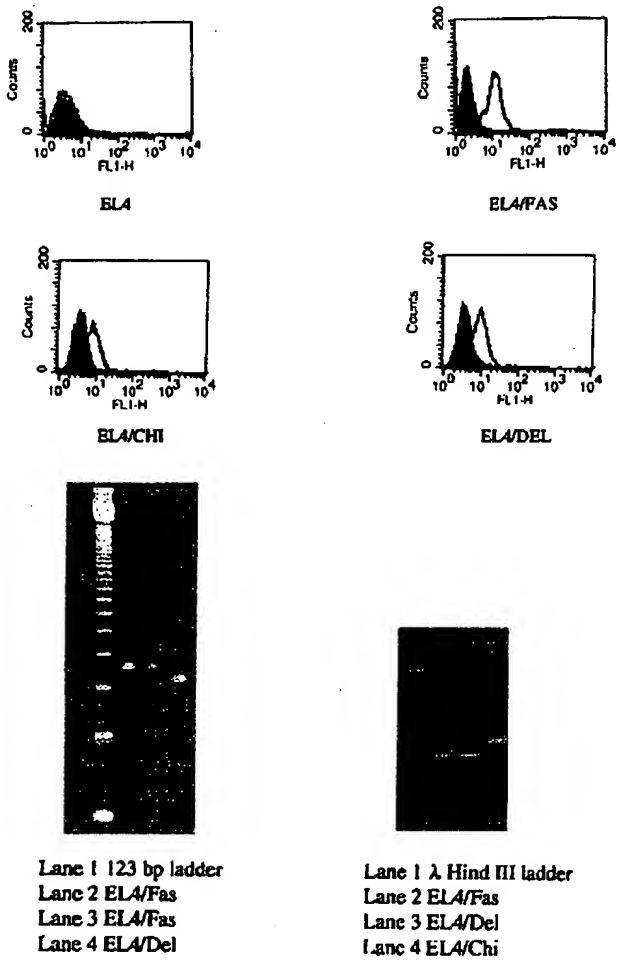



*Figure 1.* (A) Transfected EL4 cells were stained with 7C11, a mouse anti-human Fas antibody and analyzed by FACS for expression. The filled histogram shows results using secondary reagent only (FITC-conjugated goat anti-mouse), and the open histogram shows results obtained using specific primary and secondary reagents. (B) RT-PCR analysis was done on all three cell lines to verify that they encoded the appropriate receptors. PCR products generated from a 5′ forward primer from EL4/Fas and EL4/Del and a 3′ reverse primer encoding sequence from the vector were analyzed on a 5% polyacrylamide gel. A 450-bp band is expected for full-length Fas and a 405-bp band for the deletion mutant (left panel). A 5′ forward primer that includes sequence from all three constructs and a 3′ primer from the vector were used for PCR and run on a 1% agarose gel (right panel). Bands of approximately 616 bp, 571 bp, and 883 bp are expected for full-length Fas, deletion mutant, and Fas/TNFR chimera, respectively.

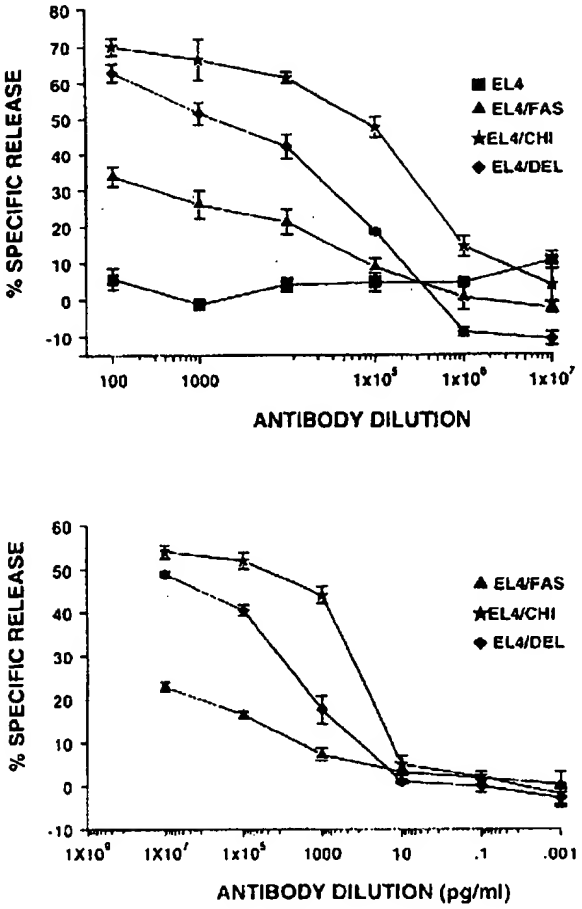



*Figure 2.* (A) $^{51}$Cr-labeled cells were incubated with various dilutions of 7C11. $^{51}$Cr release was determined after 24 h (■ = EL4, ▲ = EL4/Fas, ★ = EL4/Chi, ◆ = EL4/Del). Spontaneous release for EL4 = 25%, EL4/Fas = 32%, EL4/Chi = 34%, and for EL4/Del = 32%. (B) $^{51}$Cr-labeled cells were incubated with various dilutions of CH11. $^{51}$Cr release was determined after 24 h (▲ = EL4/Fas, ★ = EL4/Chi, ◆ = EL4/Del). Spontaneous release for EL4/Fas = 26%, EL4/Chi = 20%, and EL4/Del = 25%.

lines were sensitive to only one antibody, while others were sensitive to both or neither, regardless of equivalent expression levels. We tested our lines for sensitivity to anti-Fas antibody-mediated death with two independently derived agonistic antibodies. The deletion mutant showed enhanced susceptibility to death in accord with earlier work [6]. These data suggest that the deletion mutant has lost some regulatory component of signaling which results in increased sensitivity to death. The chimera transfectants were the most sensitive cell lines. Based on antibody dilution, it is approximately 100- to 1000-fold more sensitive than the wild-type Fas transfectants (Fig. 2) even though expression is slightly lower (see Fig. 1a). The chimera is able to signal using the intracellular portion of TNFRI simply by cross-linking the extracellular domain of Fas. Its increased sensitivity to death suggests that perhaps Fas signaling involves additional regulatory elements that are lacking in the TNFRI signaling pathway. Alternatively, TNFRI may activate additional pathways in these cells that are not activated or are activated less efficiently by Fas binding.

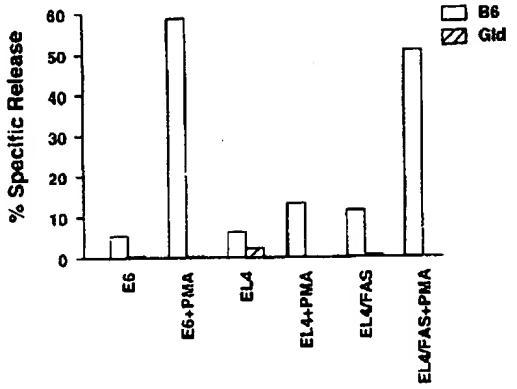


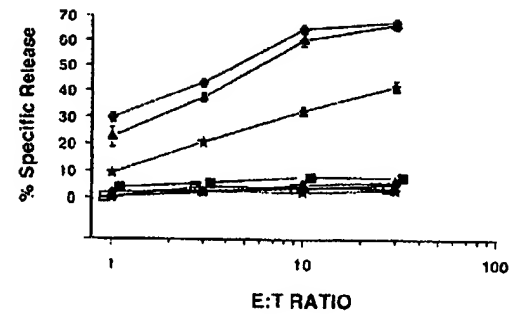

*Figure 3.* B6 anti-BALB/c T cells were used as effector cells against transfected EL4 cells. B6.*gld* anti-BALB/c T cells were used as a control to demonstrate that the lytic activity was FasL dependent. The assay consists of incubating the target cells with $^{51}$Cr for 2 h and then mixing the effectors (FasL$^+$) with the targets (Fas$^+$) in the presence of PMA. $^{51}$Cr release was measured after 6–8 h. Spontaneous release for E6 = 8.9%, EL4 = 8.5%, EL4/Fas = 7.3%.

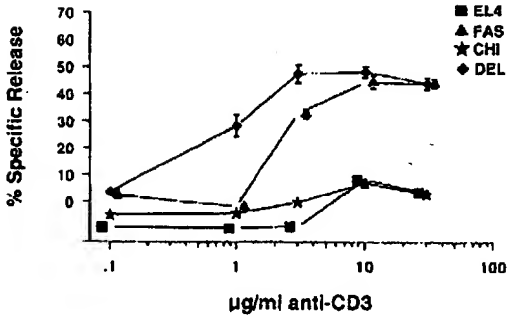
### 3.3 Sensitivity to antibody-mediated killing does not correlate with sensitivity to ligand-mediated killing

Sensitivity of the various constructs to FasL was also tested using activated B6 T cells in the presence of PMA. Activated B6 effector T cells were capable of killing E6 as well as the Fas-transfected EL4 cells in the presence of PMA (Fig. 3). PMA is required to up-regulate FasL on the effector cell and is a sufficient signal to activate the Fas-mediated pathway in long-term T lymphocyte lines (manuscript in preparation). B6.*gld* effectors which lack functional FasL were used as an additional control to demonstrate that the lytic activity was FasL dependent.

Fas and deletion mutant transfectants exhibit similar sensitivity to killing by natural ligand. In contrast, the chimera targets that are 100- to 1000-fold more sensitive to antibody-mediated death are 100-fold less susceptible to FasL-mediated lysis (Fig. 4, upper panel). Pre-incubation of the effectors alone with PMA results in cytotoxicity identical to that obtained by exposing both effector and target cells to PMA (data not shown).

To avoid pharmacological activation of the T cell, effectors were plated in wells coated with graded concentrations of anti-CD3 antibody at a constant E:T ratio. Anti-CD3 signals through the TCR to up-regulate FasL along with other proteins, allowing target killing at different levels of cellular activation, thereby providing a more direct comparison to stimulation with different antibody dilutions compared to that obtained by increasing the E:T ratio. In this assay, Fas and the deletion mutant retain their signaling capacities. The deletion mutant is slightly more potent (about threefold) at low levels of anti-CD3 stimulation. The chimera is essentially resistant to killing (Fig. 4, middle panel). This demonstrates a complete dissociation between antibody-mediated death and other forms of stimulation more representative of situations *in vivo*.



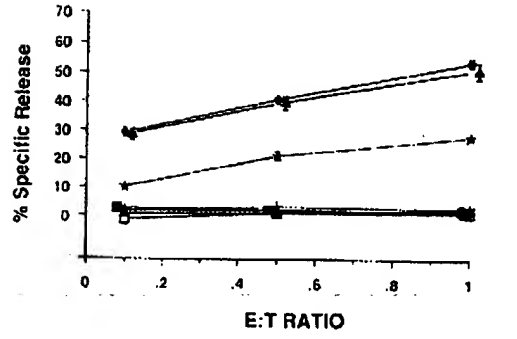




*Figure 4.* (A) B6 anti-BALB T cells were used as effector cells against transfected EL4 cells in a $^{51}Cr$ release assay. Target cells (Fas$^+$) were labeled with $^{51}Cr$ for 2 h and then mixed with FasL$^+$ effectors in the presence of PMA. $^{51}Cr$ release was measured after 6 h (■ = EL4, ▲ = EL4/Fas, ★ = EL4/Chi, ◆ = EL4/Del open = − PMA, closed = + PMA). Spontaneous release for EL4 = 10%, EL4/Fas = 6.7%, EL4/Chi = 7.1%, and EL4/Del = 5.9%. (B) B6 anti-BALB T cells were used as effector cells against transfected EL4 cells in a $^{51}Cr$ release assay. Target cells (Fas$^+$) were labeled with $^{51}Cr$ for 2 h and then mixed with FasL$^+$ effectors in the presence of bound anti-CD3. $^{51}Cr$ release was measured after 6 h (■ = EL4, ▲ = EL4/Fas, ★ = EL4/Chi, ◆ = EL4/Del). Spontaneous release for EL4 = 21%, EL4/Fas = 15%, EL4/Chi = 16%, and EL4/Del = 17%. (C) $^{51}Cr$-labeled cells were incubated with L cells transfected with a vector containing FasL in the 5′ → 3′ orientation (closed symbols) or a vector with FasL in the 3′ → 5′ orientation (open symbols). $^{51}Cr$ release was determined after 6 h (■ = EL4, ▲ = EL4/Fas, ★ = EL4/Chi, ◆ = EL4/Del open = FasL antisense, closed = FasL sense orientation). Spontaneous release for EL4 = 5.9%, EL4/Fas = 7.1%, EL4/Chi = 6.9%, and EL4/Del = 5.9%.

PMA and TCR signaling also increase intercellular adhesion through their effects on the expression of LFA-1 and possibly other accessory proteins. To determine the potential effects of these TCR or PMA-induced adhesion functions, L cells transfected with FasL were also used as effector cells. The observed order of potency is the same as with activated lymphocyte effectors, again substantiating that PMA has no direct effect on the target cells (Fig. 4, lower panel). Additionally, this confirms the different sensitivities of various constructs to agonist antibody versus endogenous ligand.

To determine whether the difference between antibody and ligand-mediated killing is not simply a matter of a membrane-aggregated agonist, we tested the effects of soluble ligand. A plasmid containing the extracellular domain of murine FasL was used to transiently transfect COS cells. [8] Supernatants were harvested and used to kill Fas, the deletion mutant, and the chimera (Fig. 5). Again, full length Fas and the deletion mutant retain their signaling capacities, while the chimera is essentially resistant to killing. It is known that this soluble ligand construct forms 10mer aggregates similar to an IgM antibody [8]. These data suggest that the major difference between antibody-mediated killing and FasL-mediated killing is not a difference in soluble versus bound stimulus nor a difference in degree of aggregation but a qualitative difference in the signals produced by physiological ligand versus agonist antibody.

## 4 Discussion

The EL4 mouse lymphoma was used to study the interaction of human Fas with mouse FasL and the ligand's ability to trigger various forms of Fas. Fig. 2 confirms earlier work that the human and mouse Fas/FasL proteins recognize one another in functional assays [10]. Two different assays, antibody versus ligand-mediated killing, demonstrate how different agonists can activate different components of a signaling pathway. The data presented here may explain why many cells lines express both Fas and

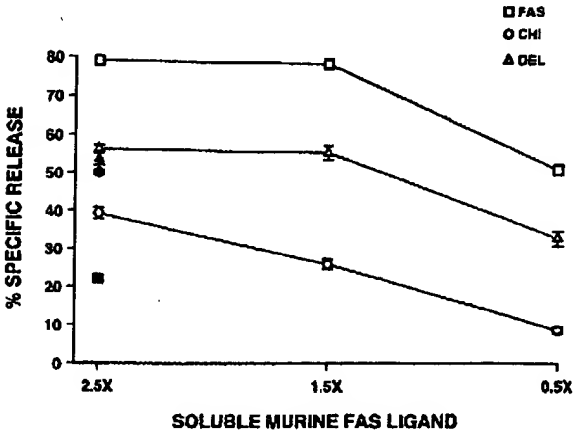



*Figure 5.* $^{51}Cr$-labeled cells were incubated with supernatants from COS cells transiently transfected with murine FasL. $^{51}Cr$ release was determined after 6 h. Spontaneous release for EL4/Fas = 18.05%, EL4/Chi = 12.83%, and EL4/Del = 12.67%.

TNFRI, but only some die in response to both anti-Fas and anti-TNFRI antibodies. Often such double-positive cell lines are resistant to apoptotic death triggered by one or both of these antibodies [11, 12]. This has led to much confusion over the similarities or differences between the two death signaling pathways.

To address this issue, we produced a chimera composed of the extracellular domain of Fas and the intracellular domain of TNFRI. In EL4 cells, the chimera was most sensitive to anti-Fas antibody-mediated death, followed by the deletion mutant, and last the full-length Fas receptor. However, when FasL was used as an agonist, the chimera was no longer as potent as the wild-type receptor, and the deletion mutant lost much of its enhanced sensitivity. Similar dissociation between sensitivity to agonist antibody and physiological ligand has been observed in B cells. B cells stimulated with CD40L in conjunction with anti-IgM antibody for 48 h were sensitive to Jo-2 antibody-mediated killing, but resistant to Th1 effector cells [13]. The fact that some killing of the chimera occurred suggests that oligomerization of the intracellular components of the TNFR is sufficient to recruit the most essential intracellular effector molecules required to initiate the Fas death pathway. However, when anti-CD3-activated effectors are used, the chimera loses almost all its signaling capabilities. One possible explanation is that the level of functional FasL protein is higher on the PMA-activated T cells and the transfected L cells than on TCR-stimulated $CD8^+$ cells.

The deletion mutant, lacking the last 15 amino acids of Fas, provided the most unexpected results. It was previously shown that in fibroblasts, this mutant receptor was more sensitive to antibody-mediated death than wild-type Fas [6]. This result was duplicated in a mouse T lymphoma, EL4. In fact, Sato et al. [14] identified FAP (Fas-associated phosphatase, a potential intracellular Fas binding protein) by the yeast two-hybrid system and showed that it binds to the last 15 amino acids of human Fas. In addition, Chinnaiyan et al. [15] showed that Fas-associated death domain (FADD), another signaling molecule also cloned by the yeast two-hybrid system bound to the deletion mutant with greater affinity than to wild-type Fas. Although enhanced sensitivity to antibody-mediated death could be shown in the deletion mutant, this increase was less evident when activated T cells or a transfected cell line expressing FasL were used as agonists. These data suggest a limited role for the last 15 amino acids of Fas in the regulation and signaling mediated by this molecule.

What, then, is the difference between the antibody and the natural ligand? It is possible that the antibody may stay bound to the receptor for a longer or shorter period of time. While this line of reasoning may explain the enhanced effect of antibody on the chimera and the deletion mutant, it does not explain why wild-type Fas is less responsive to antibody-mediated killing compared to FasL-mediated killing.

An alternative explanation takes into account a qualitative rather than quantitative change. It is possible that the three-dimensional structure formed by anti-Fas antibody-aggregated Fas differs from the structure formed upon interaction of Fas and FasL. It is known that the IgM antibody is significantly larger than FasL, making it less likely to bind the same region of Fas. In this regard, Dhein et al. [16] generated a panel of anti-Fas antibodies of different isotypes but the same variable regions and found that they had different agonist activities. Fadeel et al. [17] recently demonstrated that CH11 (anti-Fas antibody) binds to a hairpin loop between the second and third cysteine-rich domains of Fas found on the opposite side of the proposed ligand-binding region. Based on molecular modelling, it has been predicted that Fas and FasL interact such that two hydrophobic faces come together with ligand on the inside and Fas clustered on the outside [18]. The aggregate formed by antibody, particularly IgM, would cluster as many as ten Fas molecules back-to-back rather than in the face-to-face trimer predicted for ligand. This difference in clustering may contribute to the different effects of the two stimuli.

Both antibody and FasL trigger Fas, but they stimulate different components of the signaling pathway. One hypothesis for how this may work involves the death-inducing signaling complex (DISC) that associates with Fas after cross-linking [19]. DISC is made up of four cytotoxicity-dependent APO-1-associated proteins (CAP), two of which have been identified as differentially phosphorylated forms of FADD [20]. Perhaps each of these proteins serves to activate different signaling cascades, and using antibody to oligomerize Fas stimulates one of these pathways more than FasL does. In conclusion, while antibodies serve as convenient agonists, they may not always accurately reflect the relative importance of various signaling components responding to stimulation by the physiological ligand.

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